Ascidian dermatan sulfates attenuate metastasis, inflammation and thrombosis by inhibition of P-selectin

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Abstract: Background: Cancer-associated thrombosis and enduring inflammation are strongly associated with cancer progression and metastasis. Heparin is the mostly clinically used anticoagulant/antithrombotic drug, and has recently been shown to exhibit antimetastatic and anti-inflammatory activities that are linked to inhibition of P-selectin and/or L-selectin. P-selectin-mediated platelet–tumor cell and tumor cell–endothelium interactions facilitate the initial steps of metastasis. Objectives and Methods: The aim of the present study was to determine the capacity of dermatan sulfates to inhibit P-selectin and to test their potential to affect thrombosis, inflammation and metastasis in respective experimental mouse models. Results: Two dermatan sulfates isolated from the ascidians Styela plicata and Phallusia nigra, composed of the same disaccharide core structure (IdoA2-GalNAc)n, but sulfated at carbon 4 or 6 of the GalNAc, respectively, have opposed heparin cofactor II (HCII) activities and are potent inhibitors of P-selectin. The ascidian dermatan sulfates effectively attenuated metastasis of both MC-38 colon carcinoma and B16-BL6 melanoma cells and the infiltration of inflammatory cells in a thioglycollate peritonitis mouse model. Moreover, both glycosaminoglycans reduced thrombus size in an FeCl3-induced arterial thrombosis model, irrespective of their HCII activities. The analysis of arterial thrombi demonstrated markedly reduced platelet deposition after dermatan sulfate treatment, suggesting that the glycosaminoglycan inhibited P-selectin and thereby the binding of activated platelets during thrombus formation. Conclusions: Collectively, these findings provide evidence that specific inhibition of P-selectin represents a potential therapeutic target in thrombosis, inflammation and metastasis, and that ascidian dermatan sulfates may serve as antiselectin agents.

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Ascidian dermatan sulfates attenuate metastasis, inflammation and thrombosis by inhibition of P-selectin

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Summary

Background: Cancer-associated thrombosis and enduring inflammation are strongly associated with cancer progression and metastasis. Heparin, is the mostly clinically used anticoagulant/antithrombotic drug, which has recently been shown to exhibit antimetastatic and antiinflammatory activities that are linked to inhibition of P- and/or L-selectin. P-selectin-mediated platelet-tumor cell and tumor-cell endothelium interactions facilitate the initial steps of metastasis. *Objectives and Methods:* The aim of the present study was to determine the capacity of dermatan sulfates to inhibit P-selectin and to test their potential to affect thrombosis, inflammation and metastasis in respective experimental mouse models. *Results:* Two dermatan sulfates isolated from the ascidians *Styela plicata* and *Phallusia nigra*, composed of the same disaccharide core structure $[IdoA2\text{-}GalNAc]_n$, but sulfated at carbon 4 or 6 of the GalNAc residues, respectively, contain opposed HCII activities and are potent inhibitors of P-selectin. The ascidian dermatan sulfates effectively attenuated metastasis of both MC-38 colon carcinoma and B16-BL6 melanoma cells, and the infiltration of inflammatory cells in a thioglycollate peritonitis mouse model. Moreover, both glycosaminoglycans, reduced thrombus size in a $FeCl_3$-induced arterial thrombosis model, irrespective of their HCII activities. The analysis of arterial thrombi demonstrated a markedly reduced platelet deposition after dermatan sulfate treatment, suggesting that the glycosaminoglycan inhibited P-selectin and thereby the binding of activated platelets during thrombus formation. *Conclusions:* Collectively, these findings provide evidence that specific inhibition of P-selectin represents a potential therapeutic target in thrombosis, inflammation and metastasis, and ascidian dermatan sulfates may serve as anti-selectin agents.

Introduction

The relationship between hypercoagulability and cancer was first observed by Trousseau more than a century ago [reviewed in 1]. Activation of blood coagulation in cancer patients is a rather common complication that has been linked to cancer progression [2]. Tumor cells express tissue factor and secrete cytokines that contribute to a prothrombotic microenvironment, which also includes activation of platelets. The pathophysiology of thrombosis in cancer is complex, but clinical and experimental evidence indicate that platelets represent the link between coagulation and cancer progression [3-5]. Activated platelets interact with the activated endothelium and contribute to recruitment of leukocytes to inflammatory sites [6]. Platelets are a rich source of pro- and anti-angiogenic factors that upon activation may contribute to angiogenesis [7]. Furthermore, platelets support the integrity of angiogenic, inflamed [8] and tumor microvessels [5]. Experimentally induced thrombocytopenia in tumor bearing animals leads to massive hemorrhage at the tumor-stroma interface, indicating a central role of platelets in tumor vascular homeostasis [5]. Although thromboembolism and inflammation are linked to cancer in a number of different ways, accumulating experimental evidence indicates that P-selectin has an integrating role in cancer progression [9-11].

P-selectin is a member of the selectin family of cell adhesion molecules that facilitates interactions among platelets, leukocytes and endothelial cells [12]. The contribution of selectins to physiological processes such as inflammation, reperfusion injury or hemostasis are well described [12]. P-selectin is present in the storage granules of endothelial cells (Weibel-Palade bodies) and platelets (α-granules), thus enabling rapid cell-surface expression upon activation

[12]. Most selectin ligands are based on the terminal tetrasaccharide structure sialyl Lewis^x (sLe^x) [12]. During hematogenous metastasis carcinoma cells carrying sLe^x-containing mucins enter the circulation and become potential candidates for selectin-mediated interactions with platelets, leukocytes and endothelium [11]. The absence of P- and/or L-selectin leads to attenuation of experimental metastasis in different animal models, implicating selectins in cancer progression [4, 13, 14]. Formation of platelet-tumor cell emboli is largely mediated by P-selectin [4] and contribute to evasion of host responses and to colonization of distant organs [3, 15].

Accumulating evidence points to the critical role of P-selectin in cancer-associated thrombosis [16]. Elevated levels of activated P-selectin-expressing platelets have been observed in advanced stages of cancer [17]. Furthermore, the presence of tissue-factor–bearing microparticles contributing to the prothrombic state has been observed in the circulation of gastric and pancreatic cancer patients [18]. P-selectin signaling through its receptor on leukocytes, P-selectin glycoprotein ligand 1 (PSGL-1), induces the generation of tissue factor-positive, highly pro-coagulant microparticles [10, 19]. Consequently, P-selectin inhibition might have a beneficial effect on survival of cancer patients, by attenuating both hematogenous metastasis and thromboembolic complications.

Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) are commonly used for prevention and treatment of cancer-associated thromboembolism [20-22]. Currently emerging clinical evidence implicates heparin in prolonging survival of cancer patients [20, 21]. There is abundant experimental data indicating that heparins attenuate metastasis by affecting angiogenesis, heparanase, P- and L-selectins or binding of cytokines [for review see 23, 24]. These observations are further supported by findings that non-anticoagulant heparin derivatives also attenuate metastasis [25, 26]. Despite promising results, an effective and safe antithrombotic

treatment remains a very challenging clinical task in cancer patients, because of the high risk of bleeding complications and thrombotic events in heparin-treated patients [20, 22].

Previously, we reported the presence of highly sulfated dermatan sulfates (DSs) in solitary ascidians (sea squids) from the orders Phlebobranchia (*Phallusia nigra*) and Stolidobranchia (*Styela plicata*) [27]. These polymers are composed of the same disaccharide backbone, consisting of [→4IdoA(2S)β-1→3GalNAcβ-1→], but differ in the position of sulfation on the GalNAc, which can be sulfated at carbon 4 or 6 [27]. The DS from the stolidobranchia *S.plicata* is sulfated at carbon 2 of IdoAc and carbon 4 of GalNAc [27] and is a potent activator of heparin cofactor II (HCII). In contrast, the DS from the phlebobranchia *P.nigra* is sulfated at carbon 2 of IdoAc and at carbon 6 of GalNAc, and is a poor activator of HCII [27].

In the present work, we show that ascidian DSs, but not mammalian DS, attenuate hematogeneous metastasis, inflammation-induced leukocytes recruitment and thrombosis. Inhibition of P-selectin has been identified as the major biological activity of ascidian DS that affects all three processes closely associated with cancer progression.

Materials and Methods

Cell lines and reagents - Human colon carcinoma cells LS180 (ATCC, Manassas, VA) were grown in alpha-MEM media (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Invitrogen). Mouse colon carcinoma cell line MC-38, stably expressing GFP MC-38GFP [13] and mouse melanoma cell line B16-BL6 [25], were grown in DMEM with 4.5 g/l of glucose supplemented with 10% FCS medium (Invitrogen). All reagents were from Sigma (St. Louis, MO) unless otherwise stated. Unfractionated heparin – Liquemine (UFH) was obtained from Roche Pharma, Switzerland.

Dermatan sulfates - The ascidian *P.nigra* was collected in Angra dos Reis, Rio de Janeiro, Brazil; *S.plicata* was collected at Praia da Urca, Rio de Janeiro, Brazil. Dermatan sulfates (DSs), were isolated from ascidian visceras by proteolytic digestion followed by anion exchange chromatography as described previously [27]. Both ascidian DS contained 2-O sulfation on α-L-iduronic acid. While *S.plicata* had additional sulfation in 4-O position of N-acetyl-β-D glucosamine unit, *P.nigra* had sulfated 6-O position. Therefore *S.plicata* DS was designated as 2,4-DS while that from *P.nigra* as 2,6-DS. Mammalian DS was obtained from Sigma Company. Oversulfated DS was prepared from a pig mucosal dermatan sulfate [28].

Activated partial thromboplastin time assay- aPTT clotting assays were carried out as described previously [27]. Briefly, normal human plasma (90 μL) was incubated with 10 μL of DS at several dilutions and 100 μL of cephalin. After three minutes at 37^{o}C, 100 μL of 0.25 M $CaCl_2$ were added to the mixtures and the clotting time was recorded. The activity is expressed as units/mg using a standard unfractionated heparin (200 IU/mg) curve.

Inhibition of tumor cell binding to immobilized selectins – The ability of DSs to inhibit the adhesion of calcein AM labeled LS180 cells to immobilized P-selectin chimeras was examined in a serial dilution of glycosaminoglycans as described previously [25].

Platelet-tumor cell aggregation in vivo – Lungs were prepared and analyzed at 30 min or 3 h after intravenous injection of tumor cells as described previously [4]. Briefly, LS180 cells were harvested with 2 mM EDTA in PBS; labeled with Calcein AM and injected intravenously in mice with or without previous intravenous application of 100 μg of DSs or 1 mg of UFH (200 IU/mg) [4]. Lung sections were stained with rat anti-mouse CD41 antibody (Becton Dickinson, Mountain View, CA), followed by the goat anti-rat-Alexa 568-conjugated antibody (Invitrogen) and analyzed by immunofluorescence microscopy. Calcein-labeled cells present in 40 view fields of six different lung sections were scored as "associated" or "not associated" with platelets.

Experimental metastasis model – Wild type (wt) or P-selectin-deficient mice (P-sel-/-) in C57Bl/J6 background (The Jackson Laboratory) were intravenously injected either with 150'000 B16-BL6 cells or 300'000 MC-38GFP cells via the tail vein. Mice received either PBS or 100 μg of mammalian DS, mammalian oversulfated DS, ascidian 2,4- or 2,6-DS intravenously injected 10 minutes prior to tumor cell injection. Mice injected with melanoma cells were terminated 15 days later and mice receiving carcinoma cells were terminated after 28 days. PBS-perfused lungs were macroscopically evaluated for the presence of metastatic foci. GFP measurement in the lungs homogenates from MC-38GFP cells-injected mice were performed as described previously [4].

Thioglycollate-induced peritonitis – Mice were injected with 1 ml of 4% thioglycollate broth into the peritoneum. Mice were intravenously injected with PBS, P-selectin function-blocking

antibody [29], 2,4-DS or 2,6-DS (4 mg/kg) five minutes after thioglycollate injection. After 4 hours, mice were terminated and peritoneal cells harvested by injection of 4 ml of PBS containing 0.5% BSA and 1 mM EDTA. The total number of cells in peritoneal lavage was counted with a hemocytometer. The differential count of polymorphonuclear leukocytes was determined in cytospin preparations stained by Hematoxylin and Eosin.

$FeCl_3$-induced arterial thrombosis – Wt or P-sel-/- C57Bl6 mice were intravenously injected with 50 μL of 2,4-DS, 2,6-DS, P-selectin function blocking antibody (4 mg/kg) or PBS, 10 minutes before induction of thrombosis. Mice were placed in a supine position and the common carotid artery (CCA) was exposed. Thrombosis was induced by placing a Whatman filter paper saturated with 10% $FeCl_3$ in 10% glycerol on CCA. After three minutes, the $FeCl_3$-paper was removed and CCA was rinsed with PBS. Blood flow was monitored with an ultrasonic flow probe (Transonic System, USA) until complete vessel occlusion occurred [30]. For histological analysis, mice were terminated 20 minutes after removing $FeCl_3$-paper and CCA was carefully dissected and frozen in OCT compound (Tissue-Tek, Sakura). Tissue sections were stained with hematoxylin and eosin. Platelets were detected with rat anti-mouse CD41 antibody, followed by Alexa 568-conjugated goat anti-rat antibody and analyzed by fluorescence microscopy. DAPI was used for visualization of cell nuclei. At least five images acquired by Zeiss AM200 microscope were analyzed by Imaris® software per group (Bitplane AG, Zürich, Switzerland).

Results

Dermatan sulfates inhibit tumor cells binding to P-selectin

To study the structural requirements of ascidian and mammalian dermatan sulfates (DSs) for P-selectin recognition, we tested the capacity of DSs to inhibit binding of LS180 cells to P-selectin. (Figure 1A). Ascidian DSs (2,4-DS or 2,6-DS) as well as a chemically oversulfated mammalian DS (OSDS) inhibited tumor cell binding to P-selectin at comparable levels, with an IC_{50} of 13.5 μg/ml (ascidian 2,4-DS) and 12.2 μg/ml (ascidian 2,6-DS) and 12.6 μg/ml (OSDS). All disulfated DSs were superior to UFH (24.5 μg/ml). In contrast, single sulfated mammalian 4-DS from porcine skin did not inhibit P-selectin binding to tumor cells even at higher concentrations. These observations indicate that the sulfation degree of dermatan sulfate is critical for P-selectin recognition (Table 1). While ascidian DSs contain high levels of 2,4- or 2,6-disulfated disaccharides, 66% and 78%, respectively [27], mammalian DS contains mostly 4-monosulfated disaccharide units (80%). Disaccharide analysis of OSDS from porcine skin revealed a distinct sulfation pattern composed of mostly 4,6- disulfated disaccharides (35.5%) and containing small amount of 2,6-disulfated, 6-monosulfated and trisulfated disaccharides, 18.3%, 20.4% and 23.4% respectively. OSDS or ascidian DSs showed similar IC_{50} values in the P-selectin binding assays, suggesting that degree of sulfation, rather than the position of sulfate groups, is critical for P-selectin interaction. In contrast, the anticoagulant activity of ascidian DSs has been shown to depend on the position of sulfation on the N-acetylgalactosamine [27].

L-selectin also binds GAGs such as heparin and heparan sulfate [31]. Although, L-selectin binding to 2,4 and 2,6-DSs was less efficient than of P-selectin (2-fold lower), it was comparable to the binding of unfractionated heparin (Suppl. Figure 1).

P-selectin-mediated binding of platelets to selectin ligands on tumor cells significantly contributes to tumor cell emboli formation [4]. To test ascidian DSs as inhibitors of P-selectin-mediated interactions in vivo, mice were injected with calcein-labeled LS180 cells 10 minutes after application of DSs (100 μg/mouse), PBS, or UFH (1 mg/mouse). The extent of platelet-tumor cell emboli in the lung microvasculature was evaluated 30 min and 3 hours later (Figure 1B). Initial seeding of tumor cells in lungs was comparable among all groups after 30 min but the number of viable tumor cells detected after 3 h significantly decreased in mice injected either with DSs or heparin. These results confirmed that ascidian DSs are potent inhibitors of P-selectin-mediated interactions also in vivo, while the inhibitory effect of DSs lasted for at least 3 hours.

Ascidian DSs attenuate experimental metastasis

Inhibition of P-selectin or the absence of P-selectin has been previously shown to attenuate metastasis [4, 14]. To determine the ability of ascidian DSs to attenuate metastasis, wt mice were intravenously injected with 100 μg of each ascidian DS followed by injection of MC-38GFP cells. After twenty eight days, mice were terminated and lungs evaluated for metastasis (Figure 2). Both ascidian DSs (2,4-DS or 2,6-DS) markedly attenuated metastasis, when compared to PBS injected control mice with lungs displaced by metastasis (Figure 2A). Lungs of ascidian DSs-treated mice showed only a few metastatic foci, 0-7 foci per lung (Figure 2B). Injection of

mammalian DS did not affect metastasis, which is in agreement with the previous observation that 4-DS had no effect on P-selectin inhibition (Figure 1A). To confirm that the antimetastatic effect of DS is dependent on P-selectin, we intravenously injected ascidian DSs followed by MC-38GFP in P-selectin-deficient mice (P-sel-/-) (Figure 2B). While P-selectin deficiency alone markedly reduced metastasis [4, 13], small number of metastatic foci were clearly detectable (3-25 foci per lung). However, 2,4- or 2,6-DS had only minimal additional effect on metastasis, indicating that the anti-metastatic effect of ascidian DSs in wt mice depends on inhibition of P-selectin-mediated interactions.

The anti-metastatic effect of ascidian DSs was also evaluated with B16-BL6 melanoma cells, which were previously reported to express P-selectin ligands, albeit to a lesser extent than MC-38 cells [25]. Both ascidians DSs attenuated metastasis of B16-BL6, although less efficiently than by MC-38GFP cells (Figure 3).

Ascidian DSs inhibit recruitment of polymorphonuclear cells

There is accumulating evidence that inflammatory cells affect tumorigenesis and metastasis, although the underlying mechanism is still under investigation [32, 33]. Since ascidian DSs are potent P-selectin inhibitors, we tested their capacity to inhibit leukocyte recruitment in a thioglycollate-induced peritonitis mouse model. Intraperitoneal injection of 4% thioglycollate was followed by intravenous injection of 100 μg of 2,4- or 2,6-DS or P-selectin function-blocking antibody (P-sel Ab), respectively. Analysis of peritoneal lavage leukocytes showed a three-fold increase in the number of polymorphonuclear cells (PMN) in thioglycollate treated mice when compared to controls (Figure 4A). Both ascidian DSs significantly reduced leukocyte

recruitment, primarily PMNs (Figure 4B). Ascidian DSs reduced peritoneal recruitment of PMNs to the similar extent as observed in mice treated with P-sel Ab. Thus, inhibition of P-selectin and possibly L-selectin are primarily responsible for the anti-inflammatory effect of ascidian DSs.

Ascidian DSs inhibit arterial thrombosis

To test whether P-selectin inhibition by DSs affects thrombosis, we applied ascidian DSs in the $FeCl_3$-induced carotid artery lesion model. An increased time to occlusion has been observed for both anticoagulant dermatans, 2,4-DS and mammalian DS respectively (Figure 5A). Although 2,6-DS has low HCII-mediated anticoagulant activity [27], it was able to trigger a small but significant prolongation of the occlusion time. Furthermore, we observed similar prolongation of the occlusion time both in P-sel-/- mice and wt mice treated with P-sel Ab (Figure 5A). Representative normalized blood flow curves obtained from experiments with mice treated either with 2,6-DS or P-sel Ab revealed a tendency to an increase of the initial plateau, followed by a sharp drop in the blood flow (Figure 5B). Interestingly, the curve obtained in the experiments with 2,4-DS-treated mice showed not only the same prolonged initial plateau, but also a milder drop (green line). To test whether P-selectin inhibition by DSs affects thrombus formation, we analyzed the carotid arteries 20 min after the induction of thrombosis. Histological analysis revealed smaller thrombi in mice treated with 2,4- and 2,6 DS, but a marked difference in thrombi composition was observed (Figure 5C). Whereas control mice and mammalian DS-treated mice showed massive thrombi, mice treated with ascidian DSs or P-sel-Ab showed less compact thrombi. Further analysis of platelet content revealed platelet-poor thrombi in mice treated either by P-sel Ab or ascidian DSs, while platelet content in mammalian DS-treated mice

was only partially reduced (Figure 5C). These observations provide evidence that inhibition of P-selectin by ascidian DS attenuates thrombi formation by inhibition of platelet deposition.

Discussion

Cancer patients are at high risk for venous thromboembolism (VTE) and P-selectin was recently identified as an independent risk factor [16, 34]. The capacity of tumor cells to activate the hemostatic system appears to be critical not only for thrombotic events but also favors tumor progression, angiogenesis, and metastasis [2, 20]. P-selectin binding to PSGL-1 that is mainly present on leukocytes triggers, together with other mediators, the release of procoagulant microparticles from leukocytes and platelets [10]. The number of microparticles is elevated in patients with deep venous thromboembolism [35] and also in experimental mouse model of venous thrombosis [19, 36]. Here we provide evidence that inhibition of P-selectin by ascidian DS affects thrombosis (Figure 5). The anticoagulant activity of DSs is given by the enhancement of the catalytic activity of Heparin Cofactor II (HCII), a serine protease inhibitor (SERPIN) present in the plasma, which inhibits thrombin directly and selectively and reduces thrombin generation [37]. The presence of (IdoA2S-GalNAc,4S) units is essential to the anticoagulant activity of DS polymers. Accordingly, 2,6-DS presents no appreciable anticoagulant activity while 2,4-DS is highly anticoagulant (Table 1) [27]. This difference in the anticoagulant activity of ascidian DSs was confirmed by aPTT ex vivo and a stasis/hypercoagulability-induced model of venous thrombosis [38]. The 2,4-DS decreased the thrombus weight by 90% while the 2,6-DS did not have any significant effect. Further analysis on an arterial endothelial photochemical

injury model indicated that 2,4-DS as well as mammalian DS (4-DS) significantly prolonged the occlusion time in wild type mice. Interestingly, 2,6-DS treatment resulted in a visible tendency to prolong the occlusion time, despite its low HCII activity [37]. Moreover both ascidian DSs and not porcine DS, had a low but significant antithrombotic activity in HCII-/- mice. The aPTT performed with plasma from HCII-/- mice did not show any variation after treatment with mammalian or 2,6-DS and only a slight prolongation after 2,4-DS. Altogether, the antithrombotic activity of ascidians DSs may involve HCII-dependent and HCII-independent mechanisms [37]. Although the HCII-independent antithrombotic effect of ascidian DSs remains to be fully determined, the presented evidence (Figure 5) suggests that 2,6-DS inhibits P-selectin and thereby platelet accumulation, which reduces thrombi formation. Previously, prolonged time to occlusion was observed in P-selectin deficient mice using the $FeCl_3$-induced arterial thrombosis model [39]. Thrombi formed in the carotid artery of wt mice showed a higher content of leukocytes in comparison to P-sel-/- mice. Inhibition of P-selectin by function-blocking antibody decreased leukocyte accumulation and deposition of fibrin into growing thrombi in an arteriovenous shunt model in baboons [40]. Although we did not analyze the leukocyte content of thrombi, the inhibition of the P-selectin-dependent formation of large aggregates containing platelets and leukocytes might be an important mechanism for the antithrombotic effect of ascidian DSs.

Cancer patients are in a pro-thrombotic state and soluble P-selectin (sP-selectin) has been suggested to be a relevant marker of cancer-related thrombosis that strongly correlates with mortality [34]. The binding of sP-selectin to its main ligand expressed on the leukocyte surface (PSGL-1) leads to leukocyte activation, resulting in a release of tissue factor-bearing microparticles from leukocytes [41]. Such microparticles have been shown to accumulate in the

developing thrombi in a P-selectin- and PSGL-1-dependent manner [42]. Although we have shown that inhibition of platelet P-selectin affects thrombi formation, the subsequent production of microparticles by leukocytes, as well as their deposition in the forming thrombus may also be hampered by treatment with ascidian DSs.

P-selectin expressed on activated endothelium and/or platelets, has been shown to be essential for inflammatory leukocyte recruitment allowing cell tethering and rolling on activated endothelium [43]. The results obtained from the thioglycollate-induced peritonitis model showed that ascidian DS was as potent as the inhibition of P-selectin by function-blocking Ab or the absence of P-selectin in inhibition of PMN recruitment (Figure 4). Since ascidian DSs also efficiently bind to L-selectin (Suppl Figure 1), direct inhibition of leukocyte recruitment through L-selectin can be expected. In addition to selectin inhibition, ascidian-derived glycosaminoglycans (including DSs) have the potential to bind cytokines and growth factors and thereby interfere in cytokine-mediated inflammatory responses or growth factor mediated tumor progression [44]. Whether ascidian DSs contribute to a reduced leukocyte recruitment or attenuation of metastasis by displacing cytokines and/or growth factors remains to be determined.

There is accumulating evidence that P-selectin with its activity ranging from a cell-adhesion molecule to a signaling mediator [45], might be a valuable pharmacological target in cancer [4, 46], inflammation [47] and thrombosis [19]. In comparison to clinically used heparins, ascidian DS are rather abundant biological compounds. Further characterization of ascidian DS is certainly necessary, but their potential to affect thrombosis, inflammation and metastasis by specifically targeting P-selectin-mediated interactions makes them a potential agents for further therapy development.

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Figure Legends

Figure 1. Inhibition of LS180 cell binding to P-selectin *in vitro* and platelets *in vivo* A) Adhesion of LS180 cells to immobilized P-selectin chimera was measured in the presence of increasing concentrations of porcine DS (squares), chemically oversulfated DS (triangles) or ascidians, 2,4-DS (opened squares) or 2,6-DS (diamonds), respectively. Unfractionated heparin (UFH) was used as positive control (circle). Each curve is representative of three independent experiments. **B)** Number of tumor cells detected in the lungs at 30 min and 3 h after injection was analyzed in the presence/absence of ascidian DSs (100 μg), UFH (200 IU) or PBS, respectively. Platelet adhesion to intravenously injected tumor cells. The number of platelet-tumor cell aggregates is presented in percentage (in columns) of all counted tumor cells. The statistical significance of tumor-cell-platelet aggregation was determined by ANOVA analysis of variance; *** $p<0.001$.

Figure 2. Ascidian DSs attenuate experimental metastasis of MC-38GFP carcinoma cells. Wt mice or P-sel-/- were intravenously injected with ascidians DSs (100 μg) followed by i.v. injection of MC-38GFP cells (300'000) 10 minutes later. Metastasis was evaluated after 28 days. **A)** Representative examples of dissected lungs from wt mice are shown. **B)** Metastatic foci counts. **C)** Total tumor burden was quantified by GFP fluorescence measurement in the lung homogenate. The statistical significance was determined by ANOVA analysis of variance; ** $P<0.01$; *** $p<0.001$.

Figure 3. Ascidian DSs attenuate experimental metastasis of B16-BL6 melanoma cells. Wt mice were intravenously injected with ascidians DSs (100 μg), followed by injection of B16-BL6 cells (150'000) 10 minutes later. Mice were terminated after 14 days and lungs were

macroscopically evaluated. **A)** Representative images of lungs injected with PBS or ascidian DSs. **B)** Quantification of metastatic foci in lungs are shown. The statistical significance was determined by ANOVA analysis of variance; ** P<0.01.

Figure 4. PMN recruitment in a thioglycollate-induced peritoneal inflammation model is inhibited by ascidian DSs. Mice were injected intraperitoneally with 4 % thioglycollate broth 5 minutes before intravenous injection of PBS, P-sel-Ab, 2,4-DS or 2,6-DS (100 μg/mouse), respectively. After 4 h the peritoneal lavage was evaluated for the total amount of cells (B) and the percentage of PMN cells (C). PMN were identified by H&E staining of lavage cells (A). The statistical significance was determined by one-way ANOVA analysis; *** P<0.001. Bar = 20 μm.

Figure 5. Ascidian DSs reduces $FeCl_3$-induced arterial thrombosis primarily by P-selectin. Mice were intravenously injected with PBS, P-sel-mAb, porcine DS, 2,4-DS or 2,6-DS (100 μg/mouse), respectively, and 10 minutes later thrombus formation was induced by placing a filter paper soaked with 10% $FeCl_3$ on the common carotid artery (CCA). Flow was monitored with an ultrasonic flow probe until occlusion of CCA. **A)** Time to occlusion measurement in wt and P-selectin deficient mice upon treatments as shown. **B)** Representative flow curves of normalized blood flow registered during the experiment. **C)** After 20 minutes of thrombosis induction, CCAs were harvested and frozen. Representative images of arterial sections stained with hematoxylin & eosin (H&E) and platelet staining with anti-CD41 antibody (red) are shown. Bar H&E = 50 μm, Bar CD41 = 20 μm. **D)** Platelet content was quantified by Imaris software as

described in ‘Material and Methods’. The statistical significance was determined by one-way ANOVA analysis (*P<0.05; ** P<0.01; *** p<0.001).

Table 1.

Disaccharide content, anticoagulant and P-selectin inhibitory activities of DSs

Dermatan sulfate	Major disaccharide unit (%)	aPTT (IU/mg)[c]	Inhibition of tumor cell adhesion to P-selectin IC 50 (μg/ml)
Porcine DS	α-ΔHexUA-GalNAc(4S)[a] (80 %)	2[a]	-
2,4-DS	α-ΔHexUA(2S)-GalNAc(4S)[a] (66 %)	8[a]	13.51
2,6-DS	α-ΔHexUA(2S)-GalNAc(6S)[a] (75 %)	0.4[a]	12.19
OSDS[d]	α-ΔHexUA-GalNAc(4S,6S)[b] (36 %)	11.5	12.56

[a] Data from Pavão MS et al (27)
[b] Obtained by disaccharide analysis
[c] The anticoagulant activity of heparin and its derivatives was determined in human plasma samples as described (27).
[d] OSDS = oversulfated mammalian dermatan sulfate.

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Figure 1

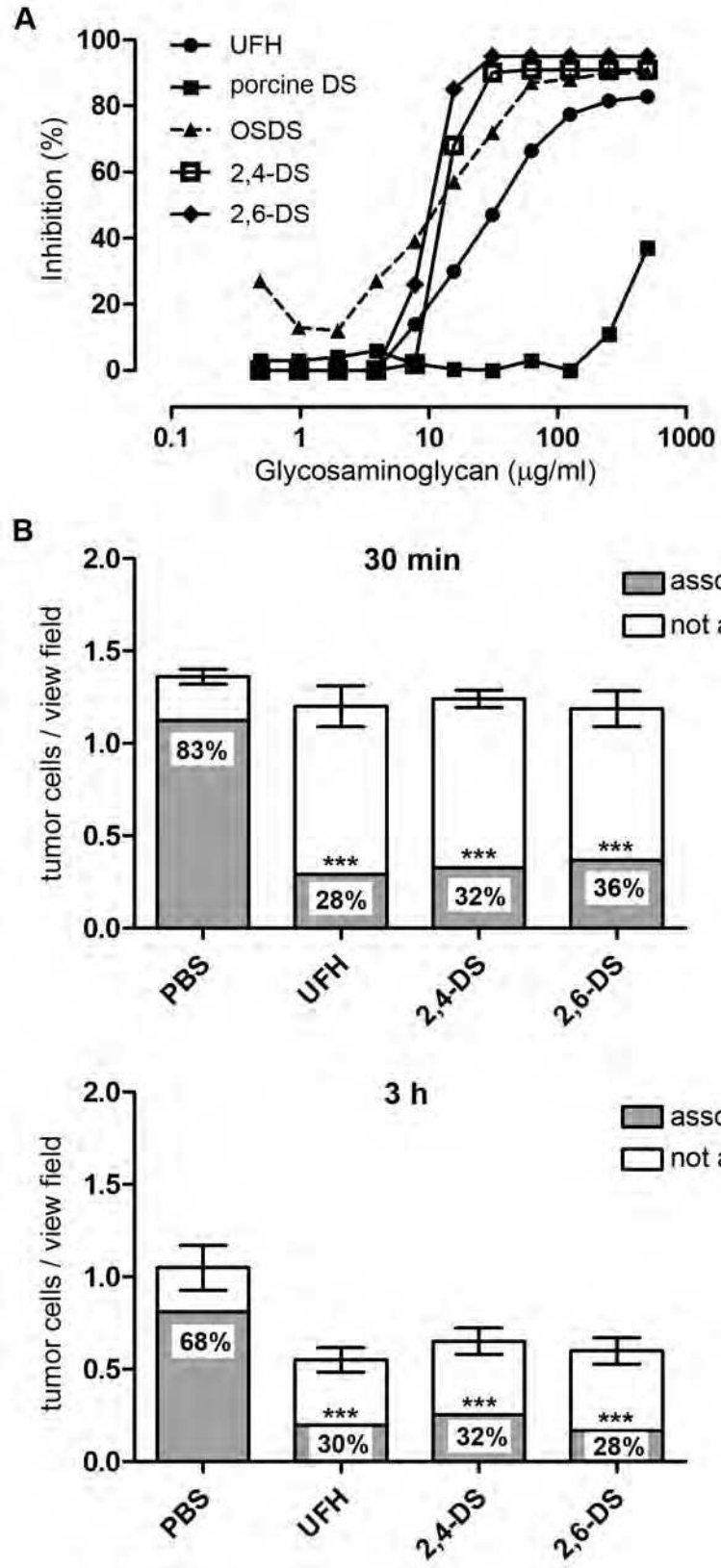

Figure 2

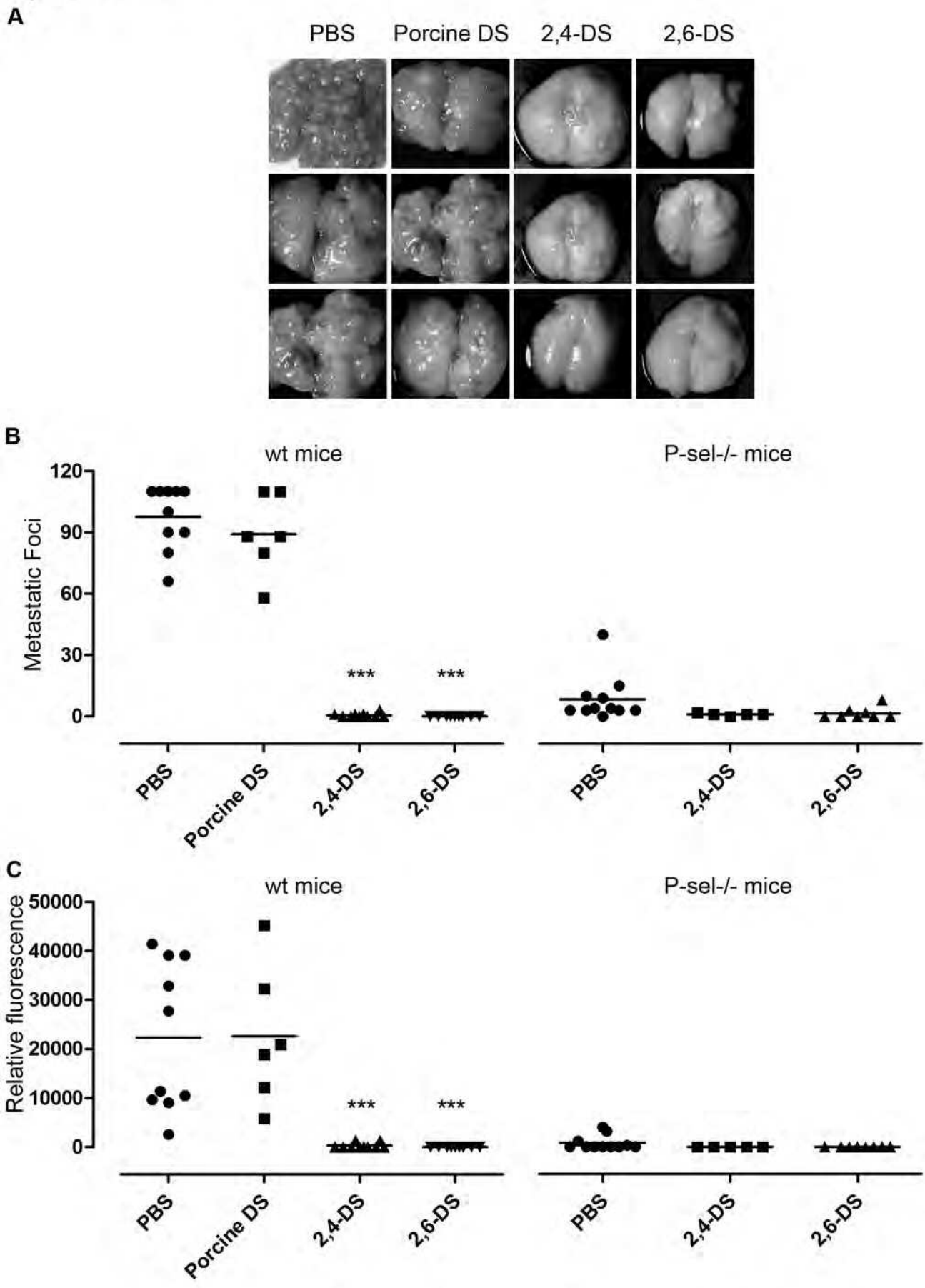
A
PBS
Porcine DS
2,4-DS
2,6-DS
B
wt mice
P-sel-/- mice
Metastatic Foci
120
90
60
30
0
***
***
PBS
Porcine DS
2,4-DS
2,6-DS
C
wt mice
P-sel-/- mice
Relative fluorescence
50000
40000
30000
20000
10000
0
***
***
PBS
Porcine DS
2,4-DS
2,6-DS

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Figure 3

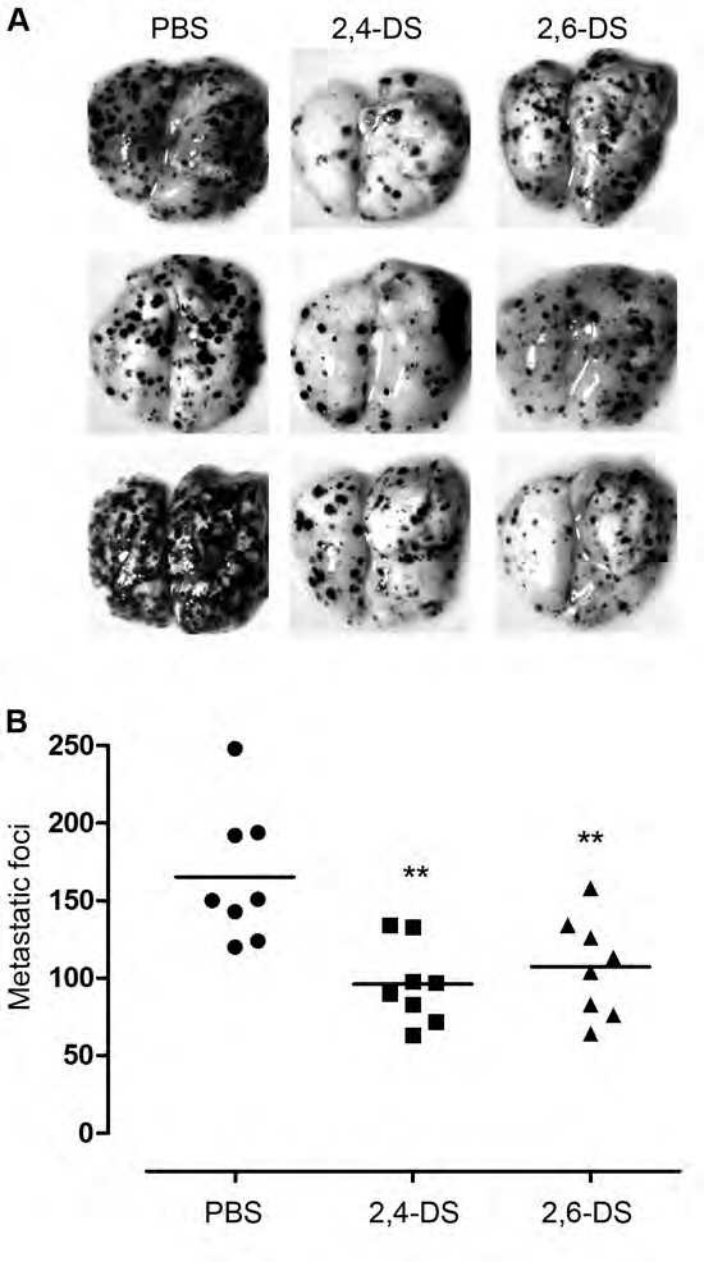

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Figure 4

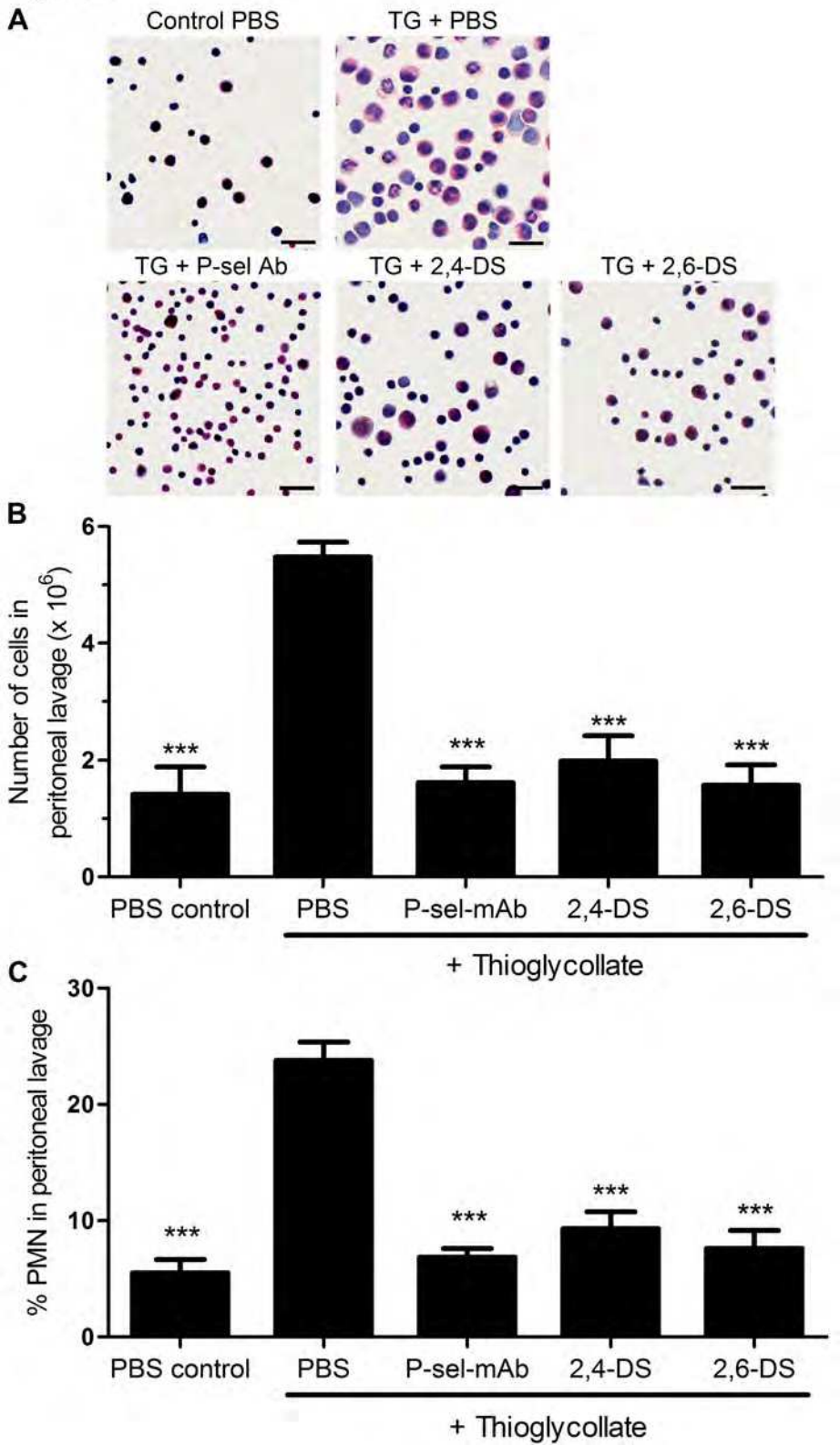

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Figure 5